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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Docket No.:	RELIA.P-113
Inventor:	Dr. Manisha DESHPANDE
Serial No.:	10/686,822
Filing Date:	October 16, 2003
Title:	TISSUEK-LIKE ORGANIZATION OF CALLS AND MACROSCOPIC TISSUE-LIKE CONSTRUCTS, GENERATED BY MACROMASS CULTURE OF CELLS, AND THE METHOD OF MACROMASS CULTURE

Mail Stop – Letter to the Commissioner – Priority Claim
Commissioner for Patents
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LETTER TO THE COMMISSIONER
PRIORITY CLAIM

In order to complete the Priority Claim in this Application, transmitted herewith is a certified copy of the Application filed in India on October 18, 2002, under Serial No. 912/MUM/202 in the name of Reliance Life Sciences Pvt. Ltd., an Indian company.

Respectfully submitted,

LACKENBACH SIEGEL, LLP
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By: 

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Certificate of Deposit by Mail

I hereby certify that this correspondence is being filed by depositing same in an envelope stamped first-class mail, addressed to the Mail Stop – Letter to the Commissioner – Priority Claim, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450, in a duly marked U.S. Postal Service drop box, with appropriate postage, on the following date:

J. Harold Nissen
Attorney

Signature

January 9, 2004
Date

Applicant hereby petitions that any and all extensions of time of the term necessary to render this response timely be granted. COSTS FOR SUCH EXTENSION(S) AND/OR ANY OTHER FEE DUE WITH THIS FEE DUE WITH THIS APER THAT ARE NOT FULLY COVERED BY AN ENCLOSSED CHECK MAY BE CHARGED TO DEPOSIT ACCOUNT #10-0100.

THE PATENTS ACT, 1970

IT IS HEREBY CERTIFIED THAT, the annex is a true copy of Application & Provisional Specification filed on 18.10.2002 in respect of patent application no.912/MUM/2002 of Reliance Life Sciences Private Limited, an Indian company, having its registered office at of Chitrakoot, 2nd Floor, Ganpatrao Kadam Marg, Shree Ram Mills Compound, Lower Parel, Mumbai 400 013, Maharashtra, India.

This certificate is issued under the powers vested in me under Section 147 (1) of the Patents Act, 1970.

Dated this 10th day of December 2003

(R. Bhattacharya.)
ASST. CONTROLLER OF PATENTS & DESIGNS

FORM I
THE PATENTS ACT, 1970
APPLICATION FOR GRANT OF A PATENT
See sec Sec 7

ORIGINAL

1. We, **Reliance Life Sciences Private Limited,** an Indian Company, having its Registered Office at of Chitrakoot, 2nd Floor, Ganpatrao Kadam Marg, Shree Ram Mills Compound, Lower Parel, Mumbai 400 013, Maharashtra, India.

2. Hereby declare:-

   a) that we are in possession of an invention titled **"Macroscopic tissue-like histologically competent constructs, generated *in vitro* by macromass culture of cells, and the method of macromass culture ".**

   b) that the provisional specification relating to this invention is filed with this application.

   c) that there is no lawful ground of objection to the grant of patent to us;

3. further declare that the inventor(s) for the said invention are:

   a) Dr. Manisha Sharadchandra Deshpande, residing at
   Flat no. 104, New Sarvodaya CHS,
   Plot 29/B, Sector 4,
   Vashi, Navi Mumbai - 400 703.
   Maharashtra,
   India

   b) Dr. Manoj Vinoy Mojamdar, residing at
   Flat no. 102, 'A' Wing,
   Naperol Towers,
   R.A. Kidwai Marg,
   Wadala, Mumbai - 400 031.
   Maharashtra,
   India

912/mum/2002
18/10/2002

all citizens of India.

912 | मुंबई MUM | 2002

18 OCT 2002

That we are the assignees of the true and first inventors.

That our address for service in India is:
Reliance Life Sciences Private Limited,
Module 9 CU 8
Old ICI Godown
Off Raey Road Station(E)
Fosbery Road
Sewree
Mumbai 400 033
Maharashtra, India

Following declaration was given by the inventors

We,

a) Dr. Manisha Sharadchandra Deshpande, residing at
Flat no. 104, New Sarvodaya CHS,
Plot 29/B, Sector 4,
Vashi, Navi Mumbai - 400 703.
Maharashtra
India

b) Dr. Manoj Vinoy Mojamdar, residing at
Flat no. 102, 'A' Wing,
Naperol Towers,
R.A. Kidwai Marg,
Wadala, Mumbai - 400 031.
Maharashtra
India

all citizens of India.

The true and first inventors for this invention declare that the applicants herein are our assignee.

Dr. Manisha Sharadchandra Deshpande

Dr. Manoj Vinoy Mojamdar

That to the best of our knowledge, information and belief the fact and matters stated herein are correct and that there is no lawful ground of objection to the grant of patent to us on this application.

Following are the attachment with the application:

a. Provisional Specification (3 copies)
b. Drawings
c. Statement and undertaking on Form 3.
d. Abstract of the invention.
e. Fee Rs.5000/- by cheque

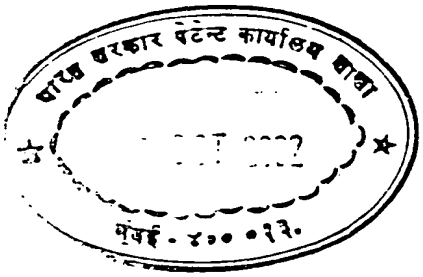
We request that a patent be granted to us for the said invention.

Dated this 18th day of October, 2002.

For RELIANCE LIFE SCIENCES PVT.LTD. . .

K.V.Subramaniam
Sr. Executive Vice President

To:
The Controller of Patents
The Patent Office
Mumbai.



FORM 2

THE PATENTS ACT, 1970
(39 of 1970)

PROVISIONAL SPECIFICATION

SECTION 10

"Macroscopic tissue-like histologically competent constructs, generated *in vitro* by macromass culture of cells, and the method of macromass culture".

ORIGINAL

RELIANCE LIFE SCIENCES PVT.LTD. an Indian Company having its Registered office at Chitrakoot, 2nd Floor, Shree Ram Mills Compound, Ganpath Rao Kadam Marg, Worli, Mumbai – 400 013, Maharashtra, India.

The following specification particularly describes and ascertains the nature of this invention and the manner in which it is performed:-

1. TITLE

Macroscopic tissue-like histologically competent constructs, generated *in vitro* by macromass culture of cells, and the method of macromass culture.

2. FIELD OF THE INVENTION

The present invention relates to *in vitro* tissue engineering. More specifically, this invention relates to the *in vitro* fabrication of three-dimensional tissue-like constructs for possible implantation in the human body as a therapy for diseased or damaged conditions.

3. BACKGROUND OF THE INVENTION

The human body can be afflicted by several diseased or damaged conditions of different organs, for which one therapeutic approach is the replacement of damaged parts, by extraneously obtained or developed tissue equivalents. For instance, burns or ulcers of the skin can be treated with application of suitable skin equivalents, non-uniting gaps in fractured bone could be treated by implantation of suitable bone substitutes, and damage to articular cartilage could be repaired by suitable cartilage-forming implants.

Every year, surgeons perform surgical procedures to treat patients who experience organ failure or tissue loss. Surgeons/physicians could treat these patients by transplanting organs from one individual to another, performing reconstructive surgery, or by using mechanical devices such as kidney dialyzers, prosthetic hip joints, or mechanical heart valves. Although these approaches have saved many lives, they are imperfect. The limitation of transplantation of organs such as the heart, liver, and kidney is not the surgical technique, but the scarce availability of donor organs.

The possible kinds of naturally available implants have been xenografts obtained from animals, allografts obtained from human donors, and autografts obtained from healthy parts of the patient itself. Xenografts have the problem of immunological non-compatibility and transmission of zoonotic pathogens including retroviruses. Allografts have the problem of immune rejection and

non-availability of donors. Autografts have the problem of lack of required amount of suitable tissue and increase in trauma to the patient.

For surgical reconstruction, tissue may be moved from one part of the patient to another part. These autografts (tissue grafts from the patient) include skin grafts for burns, blood vessel grafts for heart bypass surgeries, and nerve grafts for facial and hand reconstruction. The disadvantages of using autografts also include the need for multiple surgeries and loss of function at the donor site. In addition, surgical reconstruction often involves using the body's tissues for purposes not originally intended and can result in long-term complications.

As a result of these drawbacks of existing therapeutic options, there is a requirement for engineered tissue equivalents, and what has emerged as a new discipline is the science of tissue engineering. Its goals are to create tissues in culture for use not only as model systems in fundamental studies, but more importantly, for use as replacement tissues for damaged or diseased body parts. Although, efforts to generate bioartificial tissues and organs for human therapies go back at least thirty years, such efforts have come closer to clinical success only in the last ten years. This has been made possible by major advances in molecular and cell biology, cell culture technologies, and materials science.

The term "tissue engineering" is relatively recent and has been used more widely in the last five years to describe the interdisciplinary field that applies the principles of engineering and the life sciences toward the development of bioartificial tissues and organs.

Tissue engineering is a frontier in biomedical engineering driven by recent advances in molecular and cell biology and biotechnology. Biological and engineering tools are now becoming available to synthesize living systems at the cellular and even molecular levels, the aim of which is, to replace missing organ or tissue function caused by disease. Various innovative cell- and tissue-based therapies have recently been proposed or explored experimentally in several areas. Although a broad range of systems is studied within this thrust, common themes include: (1) the drive to understand the relationship between cell function and

the extracellular physical and chemical environment, and (2) the need to establish tools for non-destructive monitoring of tissue function.

One of the major strategies adopted for the creation of lab-grown tissues is the growth of isolated cells on three-dimensional templates or scaffolds (matrices) under conditions that will coax the cells to develop into a functional tissue. When implanted, this bioartificial tissue should become structurally and functionally integrated into the body. The matrices can be fashioned from natural materials such as collagen or from synthetic polymers such as plastics. Ultimately, the scaffold material should be biodegradable over time and should simply serve as an initial three-dimensional template for tissue growth.

As the cells grow and differentiate on the scaffold, they will produce various proteins needed to recreate a tissue. Degradation of the scaffold ensures that only natural tissue remains in the body. Cells from the patient (autologous cells) can usually be obtained from a small tissue biopsy, and these cells are desired as they avoid the risk of an immune response in the patient.

The use of autologous cells is not always feasible, however, cells from other sources can be used in combination with immunosuppressive drugs. Investigations are also underway to breed animals whose tissues would be immunologically accepted in humans. This raises ethical issues about breeding animals so that their organs can be harvested for humans and the possibility of transmitting a virus from the animal population to humans. As an alternative, genetic engineering approaches are being explored to produce universal donor cells that would not be rejected by the body and that would not pose health risks.

The major challenge in tissue engineering has been accurately imitating nature. To do this, scientists need to understand: (1) the biology of the organ to be replaced; (2) the spatial organization of the organ; (3) the biology and physical limitations of the tissues surrounding the organ; (4) the limitations of *in vitro* (laboratory) culture techniques; (5) the chemistry of creating an appropriate scaffold; (6) the physical and biochemical interactions of isolated cells with the scaffold material; and (7) the required mechanical properties of the scaffold. Scientists involved in tissue engineering efforts interact with many other scientists (biologists, biomechanical

engineers, physicians, and polymer chemists) to accomplish the seemingly impossible feat of recreating what only nature has accomplished.

Virtually every tissue in the body is a potential target for bioengineering and progress is occurring rapidly on many fronts. For the skin as an organ, different kinds of engineered replacements have been developed - skin has been re-engineered using several different approaches with varying degrees of success.

U.S. Patent No 5489304 describes a non-cellular graft which has a synthetic outer layer bonded to a collagen-chondroitin sulfate-derived dermal analog layer. This replacement, which is placed initially on the wound before a cultured epithelial autograft is applied, has the disadvantage that it lacks the growth factors important for skin wound healing or the cells that can supply these factors.

U.S. Patent No. 5460939 describes another graft, which is cellular. Here, fibroblasts are grown in bio-resorbable lactic acid / glycolic acid copolymer mesh to form a sheet. In this graft, the scaffolding mesh is not of natural origin.

Eaglstein & Falanga (1997) describe a skin graft, which includes a dermal layer having fibroblasts grown in a bovine collagen matrix. In this graft, extracellular matrix is provided extraneously to the cells, which although manufacture human collagen, but, the extraneous component remains at the time of graft application.

U.S. Patent No.5613982 describes a graft, in which human cadaver skin is processed to remove antigenic cellular components, leaving an immunologically inert dermal layer. This has the limitation of being acellular and of non-availability of human cadaver skin easily.

In all of the above examples, the technological requirements for production of the equivalents are fairly complex, hence would add to the cost of the product. Cellular sheets of fibroblasts using ascorbate have been developed, but the formation of such sheets requires about 35 days (L'Heureux *et al*, 1998).

Thus, there exists a need for the development of a dermal equivalent, the materials for which are easily available, which has no synthetic or natural extraneous matrix that could cause an inflammatory reaction in some patients, which is cellular so that it can produce growth factors and other proteins, which can be prepared in a relatively shorter time, and the preparation of which is technologically simple so that the product is more cost-effective.

An area that requires attention in the field of tissue-engineered products is bone substitutes for patients whose fractures do not heal, leaving non-uniting gaps. Autologous bone grafting increases the trauma to the patient. Different approaches are being tried in bone engineering (Service, 2000). Biomaterials such as collagen matrix infused with growth factors that trigger bone formation have been tried, but such constructs lack the cellular component and the incorporation of the required substantial amount of growth factors makes it a very expensive alternative. Ceramic or hydroxyapatite matrices seeded with mesenchymal stem cells are other approaches, but the use of such scaffolds may not be ideal for the human body. Thus, there exists a need for cost-effective cellular implants which would cause the healing of bone.

Another area that requires attention in the field of tissue-engineered products is cartilage repair. It is a known fact that articular cartilage has limited capacity for complete repair after injury. The cell-based therapy of autologous chondrocyte implantation has shown good clinical results (McPherson & Tubo, 2000) but there remains ample scope for improvement because, the time for complete repair is very long. Possibly, a pre-formed tissue rather than cell suspension would give better results upon implantation. Also, a preformed tissue has an advantage over free cells for surgical implantation. Therefore, various approaches are being tried in making a cartilage-like construct using cells and scaffold, but an ideal scaffolding matrix that will allow the cells in the implant to closely mimic the natural cartilage formation process remains a challenge (Kim & Han, 2000). Thus, there exists a need for developing a preformed tissue that could efficiently initiate cartilage repair when implanted at the site of injury, and which would also be cost-effective.

To summarize, there is a requirement for developing relatively inexpensive living cellular tissue substitutes for therapeutic purposes. Also, in general, there is always a need for the development

of tissue substitutes, which when tested, could prove to have better performance in one or more respects than existing replacements.

Looking to the need of the hour, the scientists of the present invention, have developed novel three- dimensional macroscopic tissue-like constructs which have potential to be used as tissue replacements in human body. A novel characteristic of these tissue-like constructs is that, no scaffold or extraneous matrix is used for tissue generation, the tissues are of completely cellular origin. Also, no other agents (except high cell density) such as tissue- inducing chemicals, tissue-inducing growth factors, substratum with special properties, rotational culture are employed for tissue formation. There are no specific complex medium requirements for tissue-like construct formation. The factor causing tissue formation is, large scale culture of cells at high cell seeding per unit area.

4. OBJECTS OF THE INVENTION

i. In the light of the above, it is therefore an object of the present invention to provide *in vitro* fabricated three-dimensional macroscopic tissue like constructs for possible implantation in the human body as a therapy for diseased or damaged conditions.

ii. It is another object of the present invention to provide macroscopic tissue-like constructs that are histologically competent, generated *in vitro* by macromass culture of cells and the method of macromass culture. By "histological competence" it is meant that these tissue-like constructs can be sectioned easily without disruption.

iii. It is still another object of the present invention to provide cost-effective putative tissue equivalents made from fibroblastic cells of mesenchymal origin (at least), such as an engineered putative dermal equivalent made from dermal fibroblasts, putative substitute with bone-like properties made from adipose stromal cells-derived osteogenic cells and putative substitute for cartilage repair made from

chondrocytes. It is a related object of the present invention to bring forth the possibility of providing other tissues also, which are possible to be constructed from the corresponding cell types by the method of the present invention, if these other cell types have the properties enabling them to undergo tissue-like mass formation upon macromass culture as defined in this invention.

iv. It is still another object of the present invention to provide macroscopic tissue like constructs without using scaffold or extraneous matrix for tissue generation.

v. It is still another object of the present invention to provide macroscopic tissue like constructs without using any agents such as tissue-inducing chemicals, tissue-inducing growth factors, substratum with special properties, rotational culture.

vi. It is still another object of the present invention to provide macroscopic tissue-like constructs of different kinds, formed by using high cell seeding density per unit area of culture vessel.

vii. It is yet another object of the present invention to provide macroscopic tissue-like constructs which have a high cell density in the final form.

viii. It is yet another object of the present invention to provide macroscopic tissue-like constructs which can be formed without the requirement of specific complex medium components.

ix. It is yet another object of the present invention to provide macroscopic tissue-like constructs the properties of which can be modulated to include desired properties by suitable change/s in the growth and/or tissue-forming medium.

x. It is yet another object of the present invention to provide macroscopic tissue-like constructs, which can be formed by macromass culture on different compatible growth surfaces according to requirement.

xi. It is yet another object of the present invention to provide macroscopic tissue-like constructs which can be scaled-up to larger sizes by simple scaling-up in two dimensions of the method used for their formation, viz., macromass culture.

5. DESCRIPTION OF THE INVENTION

In the present invention, there are provided macroscopic tissue-like constructs that are histologically competent, generated *in vitro* by macromass culture of cells, and the novel method of macromass culture. The present invention relates to *in vitro* tissue engineering. More specifically, this invention relates to *in vitro* fabrication of three-dimensional tissue like constructs for possible implantation in the human body as a therapy for diseased or damaged conditions.

In vitro fabrication of tissues is a goal important for the replacement of diseased tissues in the human body. Efforts are being made to explore and recruit the tissue-forming abilities of cells for *in vitro* tissue engineering.

The process of tissue engineering of cellular grafts involves the following two (2) major steps -

i. procuring the cells from suitable sources. The procured cells could require suitable preparation such as differentiation into the desired cell type.

ii. constructing the tissue using suitably prepared cells to produce different tissue engineered products.

The present invention addresses the second of these steps. The inventors have developed a simple and cost effective method for the generation of living, cellular, putative tissue substitutes.

The tissue-like constructs of the present invention have the cohesive strength to be able to withstand physical manipulation and handling as would be required for the procedure of placing

them surgically at the required site in the body from the container holding them, with the aid of appropriate supporting and handling devices or instruments.

Substantial amount of work has been done till date, in the generation of tissue substitutes that are scaffold-based – these include a scaffold as an important structural and often functional component. This scaffold requires to have properties of biocompatibility, biodegradability (so that eventually only natural tissue remains in the body) and of providing a permissive environment for optimal cellular function. The development of scaffolds that are ideal in all possible respects remains a challenge. The present invention has the advantage that it circumvents the need to incorporate a scaffold because the three-dimensional tissue-like constructs generated by the present invention are made without the aid of a scaffold. Formation of histologically competent tissue-like constructs by the macromass method of the present invention does not require a scaffold. Thus the tissue-like constructs of the present invention also eliminate any adverse effects or drawbacks that could be associated with the use of a scaffold which is anything less than ideal in any respect. In the present invention, extracellular matrix is synthesized by the cells themselves, there are no extraneous matrix components used. Tissue formation takes place simply by seeding the cells at a high cell density per unit area of culture vessel, typically 3.5 cm in diameter or larger. This has been termed as "macromass" culture which is defined as a culture system for tissue formation, in which, cells are seeded at a high density per unit area of a flat culture vessel in a range spanning a window around 10^6 cells per cm^2, the culture vessel being at least around 0.75 cm in diameter, or larger, and the cell suspension covering the full area of the vessel. The result is macroscopic tissue-like structures, wherein "macroscopic" means that the size of the tissue is *at least* such that it can be easily visually discerned by the normal human naked eye.

A previously known tissue culture system, high-density *micromass* culture has been used largely for the chondrocytic differentiation of cells, and the scale of such culture has been limited to being 10 to 20 µl spots of cell suspension (Yoon *et al.* 2000). Classically, limb mesenchymal cells when cultured *in vitro* as micromass cultures, undergo formation of precartilage condensations which are present as individual nodules covering the area of the micromass spot. The cell nodules thus formed are microscopic. The larger, yet microscopic, spheroidal structures

in which all the cells come together to form one aggregate are generated with the requirement of specific components added to the culture medium such as growth factors, as mentioned later in the text. However, the tissue-like masses generated by the present inventors are *macroscopic*, and thus possess the desirable quality of size required to have potential as tissue replacements for the human body. They also have a high cell density in the final form, which could be an added advantage for the repair process in the body.

Hitherto, the question whether cell-cell aggregation leading to macroscopic tissue-like mass formation will occur by large-scale high density culture had not been investigated. However, the work of the present inventors has addressed this question and the present invention answers in the affirmative.

High-density culture has been used to induce chondrogenesis with microscopic nodule formation, but has not been assessed so far, on the larger macroscopic scale for generation of macroscopic tissues for replacement in the human body.

Although the term "macromass" at first perception appears to mean a mere extension of "micromass", it is actually different in the important respect that micromass has been developed as a method for chondrogenic differentiation of cells and also includes specific complex medium requirements for one whole spheroidal aggregate formation (as mentioned below), while macromass is a method for the generation of macroscopic tissue-like masses or cellular aggregates, and without specific complex medium requirements for formation.

Till date, efforts have been made towards development of cellular aggregates, the results of which have been microscopic masses termed spheroids. Spheroids are three-dimensional cellular structures that have been made from hepatocytes and other cells with the help of a variety of agents like non-adherent dishes (Takezawa *et al*, 1993), spinner-flask culture (Abu-Absi *et al*, 2002), polymeric substances like Eudragit (Yamada *et al*, 1998), Matrigel (Lang *et al*, 2001), Primaria dishes (Hamamoto *et al*, 1998), poly-D-Lysine coated dishes (Hamamoto *et al*, 1998), proteoglycan coating (Shinji *et al*, 1988), culture medium flow (Pollok *et al*, 1998), rotational culture (Furukawa *et al*, 2001), liquid overlay technique (Davies *et al*, 2002), factors enhancing

cell-cell adhesion such as insulin, dexamethasone & fibroblast growth factor (Furukawa *et al*, 2001), aggregation-promoting polymer-peptide conjugates (Baldwin & Saltzman, 2001), rotating-wall bioreactor (Baldwin & Saltzman, 2001), etc. Unlike these spheroids, the tissue masses made in our work are generated without the aid of any such agent. A high cell seeding density per unit area to start with is not a formative factor for the spheroids made by the above mentioned methods. Seeding density in terms of cells per unit volume of medium is important for the above methods whereas in macromass culture, cells seeded per unit area is important. The above mentioned spheroids are much smaller, being mostly in the micrometer or sub-millimeter range. Since it is possible to make macroscopic tissue masses by macromass culture as described in the present invention, these have a clear advantage over spheroids for placement in required locations in the human body.

The largest of spheroids (about 1 mm in diameter) the present inventors have found in published literature was formed by high-density pellet culture (Mackay *et al*, 1998) using a 100-fold lower number of cells rather than by macromass culture. Their formation took place in the presence of a serum-free defined medium containing TGF-β3, dexamethasone, ascorbate 2-phosphate and insulin-transferrin-selenium supplement where as such a defined medium is not required for tissue generation by macromass culture. In the preceding report using pellet culture of bone-marrow derived mesenchymal progenitor cells, it had been found that spheroidal aggregate formation did not take place in the pellets incubated in DMEM + 10 % FCS (Johnstone *et al*, 1998). Spheroid formation by micromass culture of multipotential mesenchymal cells has been reported; here again spheroidal aggregate formation took place only upon treatment with TGFβ1 (Denker *et al*, 1995). Microscopic bone cell spheroids have been reported to form in the presence of serum-free medium containing TGFβ1 (Kale *et al*, 2000). The tissue masses of the present invention, generated by macromass culture, are much larger than any spheroids that have been developed, and have no such specific complex medium requirements for formation. A simple medium such as DMEM + 10% FCS suffices for tissue-like construct formation by macromass culture.

In one embodiment of this invention, a tissue-like sheet is formed from human dermal fibroblasts as a potential dermal substitute. The dermal fibroblasts can be of allogeneic origin, since it is

known that human dermal fibroblasts are relatively non-immunogenic upon transfer to an allogeneic recipient (U.S. Patent no. 5460939). This tissue-like sheet has the potential to be a dermal equivalent, the materials for which are easily available, which has no synthetic or natural extraneous matrix that could cause an inflammatory reaction in some patients, which is cellular so that it can produce growth factors and other proteins, which can be prepared in a relatively shorter time, and the preparation of which is technologically simple so that the product is more cost-effective.

In another embodiment of this invention, by macromass culture, a tissue-like mass with bone-like properties is generated from adipose stromal cells-derived osteogenic cells, as a putative tissue substitute that could have the potential to cause healing of small non-uniting gaps in bone fractures. This could be a possible autologous therapy. This tissue-like mass could have the potential to be a cost-effective cellular implant, devoid of extraneous scaffold, which could cause the healing of small gaps in bone.

In yet another embodiment of the invention, a tissue-like sheet has been developed from human chondrocytes, as a putative implant inducing cartilage repair in patients with articular cartilage damage. Autologous chondrocytes could be used for this tissue-therapy, since chondrocytes can be obtained from small biopsies of cartilage. This tissue-like sheet could have the potential to be a preformed tissue that could efficiently initiate cartilage repair when implanted at the site of injury, and which would also be cost-effective.

To summarize, tissue-like constructs of the present invention, generated by macromass culture could have the potential to be living, cellular, tissue substitutes that are free of scaffolds and of extraneous extracellular matrix, and that are technologically simple to make and hence would be cost-effective.

6. BRIEF DESCRIPTION OF THE DRAWINGS :

Preferred embodiments of the present invention are further illustrated in the accompanying figures, as described below -

FIG 1. Photographs showing the macroscopic tissue-like constructs formed by macromass culture, in 3.5 cm dishes, from (a) dermal fibroblasts (b) adipose stromal cells (c) chondrocytes.

FIG 2. Cell-cell adhesion process resulting in tissue-like construct formation taking place in macromass culture of adipose stromal cells (a) One hour after start of macromass culture (b) Six hours after start of macromass culture.

FIG 3. Histological examination of tissue-sheet formed by macromass culture of dermal fibroblasts (a) Hematoxylin & eosin staining shows the three-dimensional organization (b) Masson-Trichome staining shows collagen formation.

FIG 4. Histological examination of tissue-like construct formed by macromass culture of osteogenic cells derived from adipose stromal cells (a) Hematoxylin & eosin staining shows three-dimensional organization (b) Masson-Trichome staining section includes focal region of actual bone formation and the arrow points to it (c) Von Kossa staining shows focal regions of calcium deposition, marked by arrows.

FIG 5. Histological examination of tissue-like construct formed by macromass culture of chondrocytes (a) Hematoxylin & eosin staining shows three-dimensional organization (b) Masson-Trichome staining (c) Alcian blue pH 2.5 staining shows the presence of mucins.

FIG 6. Cells regrown from tissue sheet made from dermal fibroblasts by macromass culture for assessing viability.

FIG 7. Gene expression analysis of tissue sheet formed from dermal fibroblasts by macromass culture, assayed by Reverse Transcriptase-PCR. The RT-PCR products corresponding to various genes known to be important for the wound healing process of the skin are shown electrophoresed on 2 % agarose gel (M) DNA molecular size marker (1) Collagen type I (2) Syndecan 2 (3) Tenascin-C (4) Vascular endothelial growth factor (5) Collagen type III (6) Fibronectin (7) Keratinocyte growth factor (8) Transforming growth factor !β.

Various other aspects of the invention are described in further details in the following sections.

7. MATERIALS AND METHODS :

It is clarified by the inventors of the present invention that, throughout the entire description of this invention, although area of culture vessel has been referred to in terms of diameter of a circular culture vessel, the aspect being described actually includes a culture vessel of any shape, its area being same as that of a circular vessel of the mentioned diameter.

7.1. Cell isolation and culture -

In the present invention, human dermal fibroblasts were isolated from human skin biopsies. The dermis was separated from the epidermis by treatment with Dispase (Sigma, St. Louis. USA). The dermis was minced and digested with 0.01 % collagenase in DMEM + 10 % FCS overnight and then cells were allowed to attach. Cells were cultured in DMEM + 10 % FCS at 37°C in 5% CO_2 and subcultured using Trypsin-EDTA solution. Adipose stromal cells were isolated from human liposuction material according to the protocol described by Zuk *et al* (2001). These cells were maintained in DMEM + 10 % FCS. These cells were induced into osteogenic differentiation according to the protocol described by Zuk *et al* (2001). Chondrocytes were isolated from human cartilage fragment by mincing the cartilage and treating with collagenase before incubating in the maintenance medium of DMEM + 10 % FCS.

7.2. Tissue-like construct formation -

Formation of tissue-like constructs was achieved by macromass culture, which is the novel method of the present invention, earlier defined in the present description of this invention. Cultured cells were harvested using Trypsin-EDTA. They were resuspended in appropriate volume of medium and seeded typically in culture dishes with a well diameter of 3.5 cm at a seeding density of about 10^6 cells per cm^2. Thus, typically, about 10^7 cells total were seeded

in a single well of a six well plate (9.6 cm^2 area) for the formation of a single tissue. Upon formation, tissue-like constructs were fixed and processed for histological examination.

7.3. Histological analyses -

Von Kossa staining and Alcian blue staining were done on the appropriate tissue constructs of the present invention, according to the methods described by Zuk *et al* (2001) and Bancroft *et al* (1994). Oil Red O staining was done by the method described by Bancroft *et al* (1994). Other histological procedures were performed by the Histopathology Laboratory at Sir Hurkisondas Nurrotamdas Hospital & Research Centre, Mumbai, India.

7.4. Viability of cells in tissue-like constructs formed -

The tissue masses of the present invention were minced, digested with 0.5 mg/ml collagenase in serum free DMEM for 15 mins, and the released cells were resuspended in growth medium. An aliquot was stained with Trypan Blue. The cells were seeded in a culture flask to assess viability.

7.5. Gene expression analysis -

Gene expression in the tissue-like construct made from dermal fibroblasts, was analyzed by Reverse Transcriptase-PCR. RNA was extracted from the tissue-like construct using TRIZOL (Gibco-BRL, Grand Island, USA). RT-PCR was performed using primers specific for the respective genes and the Titan One-Tube RT-PCR system (Roche, Mannheim, Germany).

8. RESULTS AND DISCUSSION:

8.1. Formation of tissue-like constructs -

By macromass culture, tissue-like masses (FIG 1) were formed from dermal fibroblasts in the shape of a sheet which either detached from the growth surface spontaneously or by

gentle peeling with a blunt instrument. The adipose stromal cells also formed a tissue-like mass which was negative for lipids by Oil Red O staining. Since this was the case, to make possibly useful tissue from adipose stromal cells, they were differentiated into osteogenic cells, which upon macromass culture formed a similar sheet which can contract to a tight mass upon further incubation after detaching. Chondrocytes isolated from human cartilage also formed such a tissue sheet upon macromass culture. Cell-cell adhesion appears to be playing an important role in such tissue-like construct formation as seen from the extensive formation of extensions from the cells and cell integration, shown in FIG 2. When dermal fibroblast macromass culture was scaled up by seeding cells at the mentioned density in a 8.5 cm petri dish, a much larger sheet formed. Thus, it appears that macromass culture can be directly scaled up areawise to obtain as large tissues as desired. Dermal fibroblasts also formed a sheet by macromass culture in serum free DMEM, but the time of formation was greater than in DMEM containing serum, overnight compared to 3-4 hours in serum-containing medium. In serum-containing medium, the time of formation of tissue from dermal fibroblasts, from adipose stromal cells & osteogenic cells derived from them was about 4 hours, and from chondrocytes was about 18 hours. The tissue forming medium for dermal fibroblasts, adipose stromal cells and chondrocytes was DMEM + 10 % FCS. Osteogenic cells derived from adipose stromal cells formed a tissue-like mass in osteogenic medium as well as in DMEM + 10% FCS, after washing the cells to remove osteogenic medium.

Although these tissue-like constructs are not fully formed tissues, they could be capable of inducing and participating in the healing process in the body, in a way analogous to the findings that implantation of even stem cells or partially differentiated cells (which are not fully formed tissue but are at the very beginning of tissue formation) can lead to repair and regeneration (Kaji & Leiden, 2001).

It was found that the phenomenon of tissue-like mass formation by macromass culture was dependent on cell seeding density. To examine whether tissue formation took place at all high densities or not, dermal fibroblasts were seeded at a range of different cell densities per unit area. It was found that tangible sheet formation took place at about 3.33×10^5 cell per

cm^2, while at a density of 6.66×10^4 cells per cm^2 (five times lesser) or lower, no tissue sheet formed. Also, tissue sheet formation occurred at the density of 3×10^6 cells per cm^2 but not at 7×10^6 cell per cm^2 or higher; at the latter density the cells only loosely clumped together but did not form a cohesive tissue mass. Thus, tissue sheet formation took place at 3.33×10^5 cell per cm^2 and at 3×10^6 cells per cm^2 as well as all densities lying between these two figures that were tested. At the densities tested above or below this range, tissue formation did not occur. These data indicate that a minimum and maximum cell density per unit area exist for tissue formation by macromass culture. The range of high cell densities at which tissue formation occurs by macromass culture could be different for different cell types.

8.2. Histology (FIGS 3,4,5) -

Hematoxylin& eosin staining of the sections of the different tissue-like constructs of the present invention shows three-dimensional structural organization and extracellular matrix formation. Masson-Trichome staining of tissue-like constructs formed from dermal fibroblasts as well as native stromal cells and osteogenic stromal cells showed collagen synthesis. Thus, extracellular matrix formation also appears to be contributory to tissue-like construct formation by the method of macromass culture. There were focal regions of actual bone formation in the sections of tissue-like mass made from adipose stromal cells derived osteogenic cells, and Von Kossa staining of the tissue mass formed from osteogenic stromal cells showed focal regions of calcification, thus demonstrating bone-like properties in the tissue-like construct formed from osteogenic cells derived from adipose stromal cells. Thus, this tissue-like construct has potential as an implant that could participate and contribute to bone repair in the human body. Alcian blue pH 2.5 staining of the tissue sheet formed from chondrocytes was positive for acid mucins, which are components of the cartilage matrix.

8.3. Viability of cells in tissue-like constructs formed -

Cells re-isolated from the tissues formed from dermal fibroblasts and chondrocytes had viability greater than 98 % by Trypan Blue staining; the cells from tissue mass from adipose

stromal cells were 90% viable. The isolated cells and clumps were plated to assess regrowth, and were found to be viable in each case, as depicted in FIG 6 showing cells growing out of clumps of dissociated dermal fibroblast tissue sheet.

8.4. Expression analysis of tissue sheet formed from dermal fibroblasts -

To assess whether the tissue sheet formed from dermal fibroblasts has potential as a dermis substitute, the expression of genes known to play an important role in the wound healing process of the skin was analyzed (FIG 7). Collagen type I, collagen type III, keratinocyte growth factor, TGFβ1, fibronectin, vascular endothelial growth factor, tenascin-C and syndecan-2 were found to be expressed in the tissue sheet, thus demonstrating that this tissue-like sheet made from dermal fibroblasts has potential as a dermis substitute.

8.5. Growth surface for macromass culture -

The sheet formed from dermal fibroblasts plated on plastic surface had a tendency to detach spontaneously and curl or roll up, which is not desirable, since the sheet does not straighten once rolled up. A tissue being developed as a possible dermis substitute requires to remain straight. For this, macromass culture of dermal fibroblasts was done on a Hybond-N (Amersham Pharmacia Biotech, Buckinghamshire, UK) filter placed in a plastic dish. With this adaptation, the sheet that formed remained straightened and adhered to the filter, so, in the future, it could be applied onto a skin wound with the filter side up. This experiment demonstrates that it can be possible to achieve tissue formation by macromass culture on different compatible growth surfaces. Thus, in this case, the sheet made by macromass culture becomes a component of a putative implant that includes a nylon filter as a supporting layer. This supporting layer is not designed to integrate into the body along with the dermis-like sheet, and so it is not a scaffold which would become a part of the body, but a supporting handling device for the application of the dermis-like sheet. This supporting layer would be removed after healing.

9. CONCLUDING REMARKS:

The novelty of the present invention lies in the fact that high-density culture can be scaled up areawise to generate macroscopic tissue-like constructs of different kinds, besides in the other important features such as the fact that scaffolding material is not employed or specific agents/complex media formulations are not required, as are detailed in this description. In plainspeak, the inventors of the present invention are the first to report that macroscopic tissue-like constructs of different kinds can be generated by high-density culture.

In macromass culture, it is possible to tailor the properties of the tissue formed by including appropriate medium components or by changing the medium, since, as far as has been tested, tissue formation is independent of at least a set of medium conditions. This is, apparent from tissue sheet formation by dermal fibroblasts in both serum-containing and serum free medium, as well as from tissue formation from adipose stromal cells in both DMEM + FCS and osteogenic medium. Thus, the properties of the tissue formed can be modulated to incorporate desirable properties by modifying the tissue-formation medium and/or growth medium of the cells. For instance, as presented in this invention, bone-like properties were induced in tissue formed from adipose stromal cells by culturing the cells and forming the tissue-like construct in medium containing osteogenic components. Thus, even as it remains that tissue formation by macromass culture does not have specific complex medium requirements, such specific components can be used in the medium for modulating the properties of the tissue formed.

It may be possible that other mesodermal cells or cells of endodermal or ectodermal origin can also form such tissue-like masses by macromass culture. Preliminary experiments with a hepatoma (endodermal) and a glioblastoma (ectodermal) cell line shows that these cells also undergo a cell-cell adhesion phenomenon when cultured at similar high seeding densities at large-scale. Hence, macroscopic tissues made by macromass culture from any type of mammalian cells, if they are possible, are within the scope of this invention, the defining feature being tissues made by the method of macromass culture.

Thus, in the foregoing description, specific embodiments of this invention have been described : examples have been presented with respect to the aspects of use of different cell types, the use of alternative growth surface, the use of change in medium to modulate the properties of the tissue formed, the scale-up of tissue formation, the range of tissue-forming high cell densities, and the generation of a putative implant of which tissue made by macromass culture is a component. Although, only the described embodiments have been brought forth, they serve the purpose of example or illustration only, and do not limit the invention.

It should remain understood that different modifications or substitutions could be made to this invention, which would be within the scope of the present invention. For instance, it is contemplated by the inventors that one such modification would be the entrapment or encapsulation of tissue masses made by macromass culture into a suitable gel or matrix, or another such modification would be a construct in which multiple tissue-like masses or sheets are joined or held together by some means. In such constructs, the tissue-like construct made by macromass culture would now be a component of the whole substitute. Other modifications would be the use of other cell types which have the properties to form a tissue-like mass by macromass culture, use of other compatible growth surface than described, other medium changes for modulation, and other sizes of scale-up. Therefore, this description of the present invention is not intended to limit this invention by the precise illustrative embodiments that are disclosed.

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Dated this 18th day of October, 02

FOR RELIANCE LIFE SCIENCES PVT LTD

K.V.Subramaniam

Sr Executive Vice President

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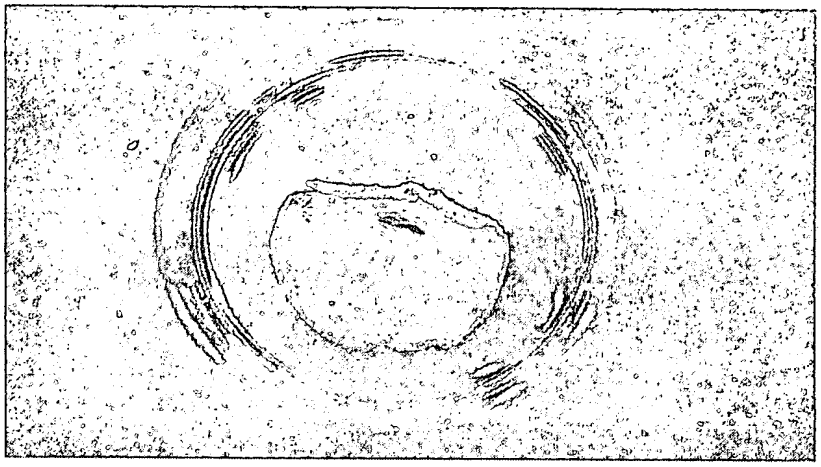
FIG 1.

a

Dermal

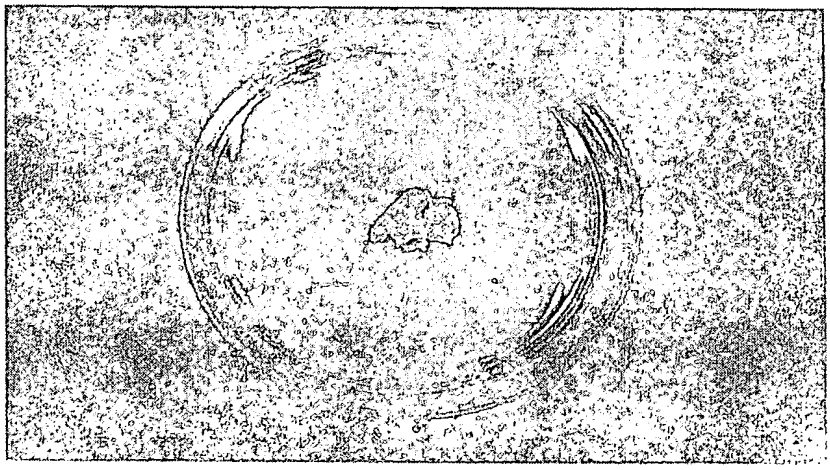
Fibroblasts



b

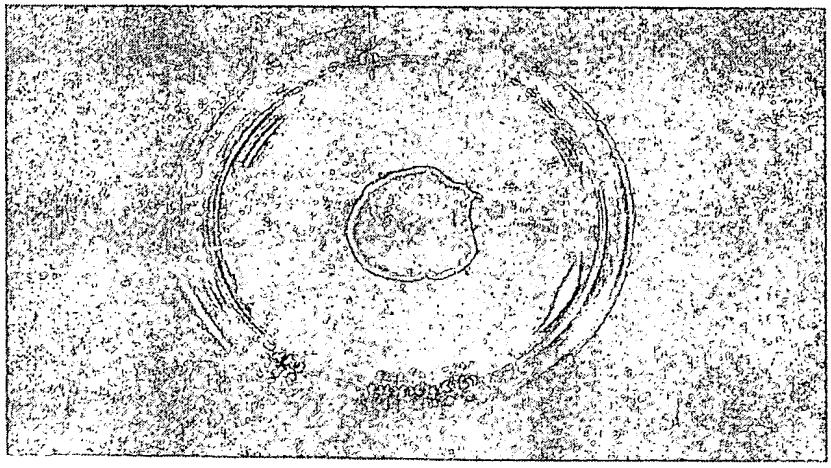
Adipose

stromal cells



c

Chondrocytes



1 cm

Tissue-like constructs formed by macromass culture of different cell types, shown in 3.5 cm dishes.

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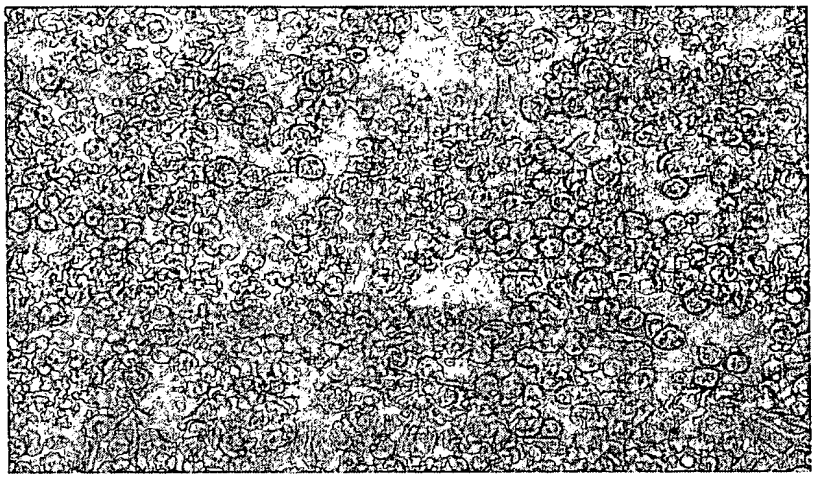
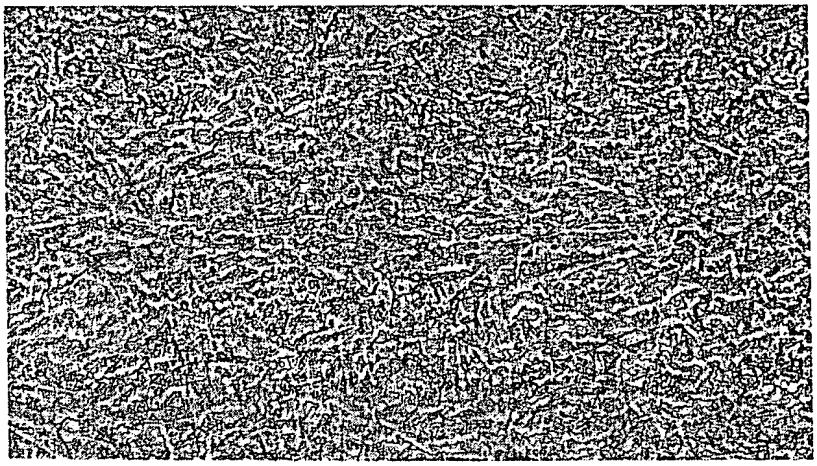
FIG 2.

a
One hour
after
incubation



b
Six hours
after
incubation



Cell-cell adhesion process in macromass culture of adipose-derived stromal cells.

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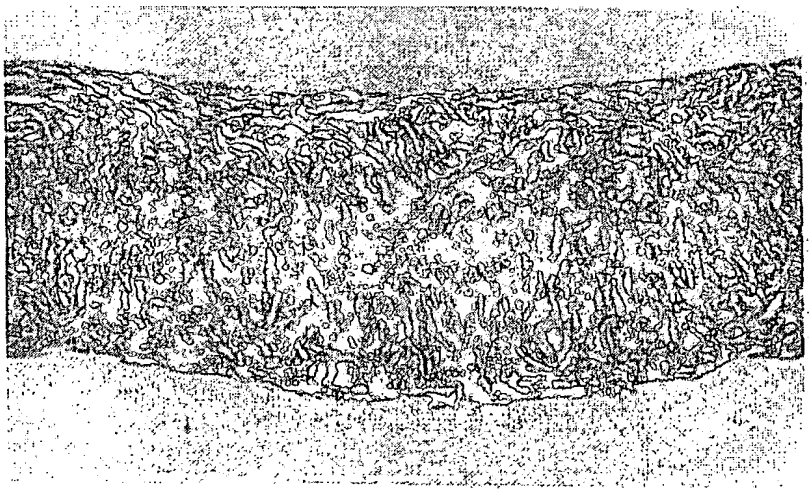
FIG 3.

a Hematoxylin & eosin

b Masson-Trichome



Histological examination of tissue-sheet formed by macromass culture from dermal fibroblasts.

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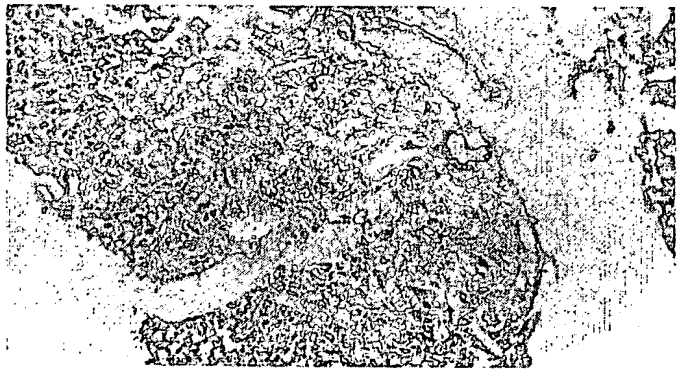
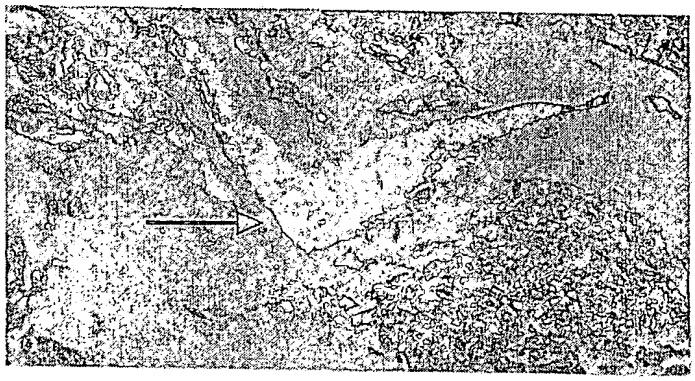
FIG 4.

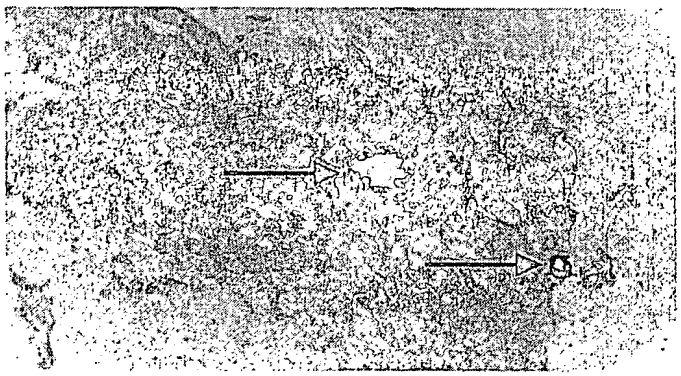
a
Hematoxylin
& eosin



b
Masson-
Trichome



c
Von Kossa



Histological examination of tissue-like construct formed by macromass culture of osteogenic cells derived from adipose stromal cells.

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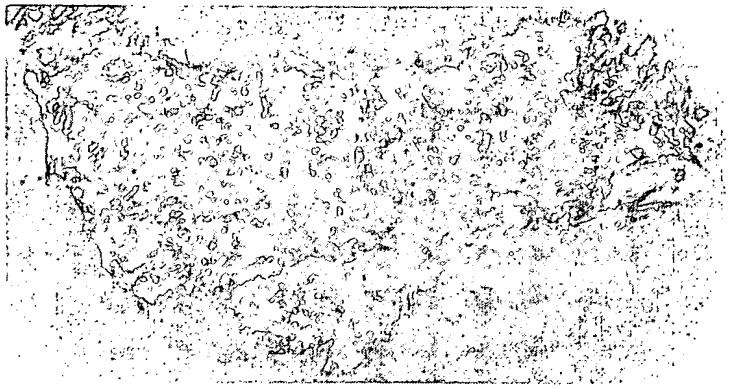
FIG 5.

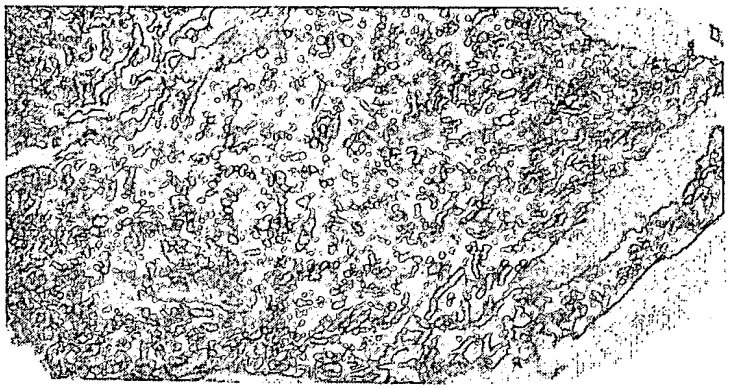
a

Hematoxylin & eosin



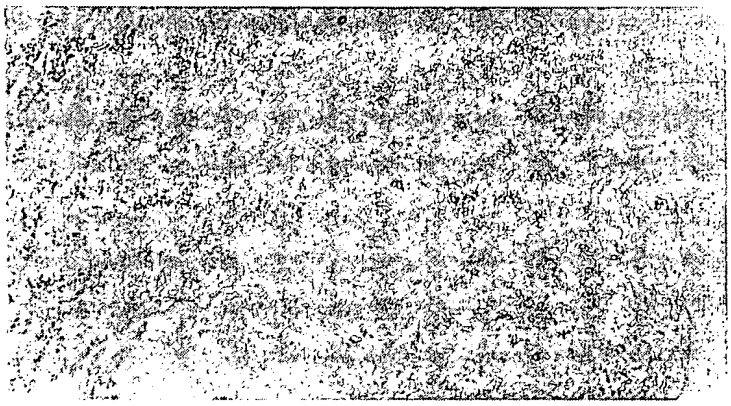
b

Masson-Trichome



c

Alcian blue pH 2.5



Histological examination of tissue- like construct made by macromass culture of chondrocytes.

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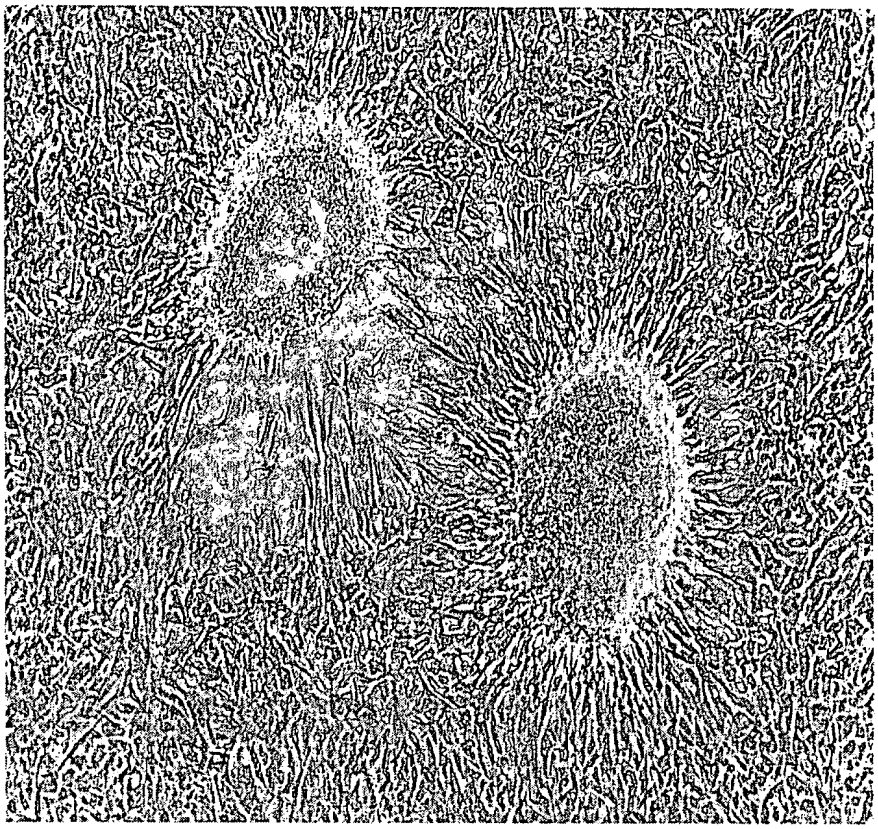
FIG 6.



Cells regrown from tissue sheet made from dermal fibroblasts for assessing viability.

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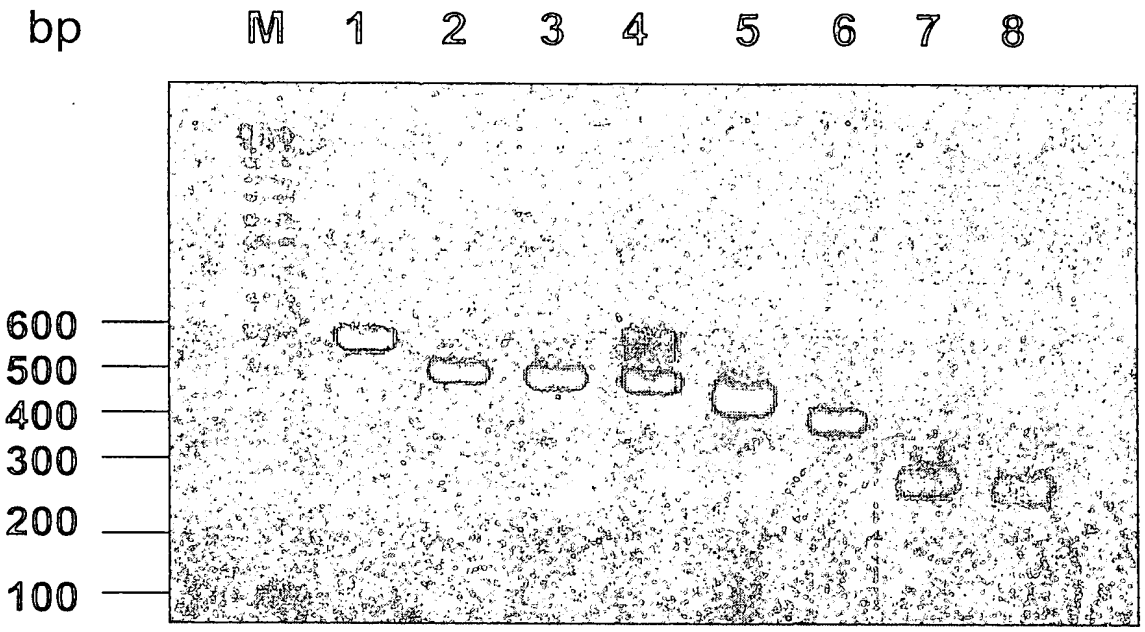
FIG 7.



M – DNA molecular size ladder
1 – Collagen type I
2 – Syndecan 2
3 – Tenascin-C
4 – Vascular endothelial growth factor
5 – Collagen type III
6 – Fibronectin
7 – Keratinocyte growth factor
8 – Transforming growth factor 1β

Gene expression in tissue sheet formed from dermal fibroblasts by macromass culture.

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